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Subtleties among subtilases

The structural biology of Kex2 and furin-related prohormone convertases

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Prohormone processing was discovered 35 years ago with the observation that proinsulin is the precursor of both the A and the B chains of insulin (Steiner, 1969). During the next 15 years, many candidate proteases were proposed and rejected before genetic and biochemical evidence identified specific processing proteases in *Saccharomyces cerevisiae* that are involved in α-factor biosynthesis (Julius *et al*., 1983, 1984). The initial cleavages of the precursor—endoproteolytic scissions carboxyl to Lys-Arg sites—are due to the action of Kex2 protease. Mammalian Kex2 homologues, known as furin, PC1/3, PC2, PC4, PACE4, PC5/6 and PC7/LPC were cloned throughout the 1990s. They were shown to process hormone and neuropeptide precursors, growth factors, receptors, metallo- and aspartyl proteases, envelope glycoproteins of many viruses including HIV, and bacterial toxins such as anthrax-protective antigen (Zhou *et al*., 1999; Thomas, 2002). These Kex2-homologous prohormone convertases (PCs) are unrelated to the tryptic enzymes of the blood coagulation cascade that hydrolyse precursors carboxyl to basic amino acids. By contrast, PCs are related to degradative enzymes, such as subtilisin and proteinase K (Fuller *et al*., 1989). Thus, accounting for the refined functions and specificity of PCs has been a major research problem. Molecular-genetic studies have established a 'P-domain' carboxyl to the subtilisin domain that is essential for the biosynthesis and/or activity of PCs. Biochemical analyses have further indicated that PCs are calcium-dependent and contain several specificity sites that confer substrate discrimination. The recent publication of the crystal structures of the secreted, soluble forms of yeast Kex2 (Holyoak *et al*., 2003) and murine furin (Henrich *et al*., 2003), each bound to specific peptidyl inhibitors, have brought these features into focus and accounted for the subtleties of these refined subtilases. Moreover, the structural coordinates may accelerate the development of inhibitors to target the bacteria and viruses that rely on processing by these proteases for infectivity.

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PCs are multidomain proteins that are targeted to late secretory compartments. Carboxyl to the signal sequence is an 80–90 amino-acid prodomain that is autocatalytically removed during maturation (Germain *et al*., 1992) in a process that involves acid-induced dissociation and cleavage at a second, internal pro-domain site (Anderson *et al*., 2002). From residue 144 to 438 (numbering according to Kex2), PCs contain a catalytic domain ~30% identical to subtilisin. Carboxyl to the subtilisin domain is the essential P-domain (Gluschankof & Fuller, 1994), followed by isozyme-specific features that are primarily involved in localization (Zhou *et al*., 1999). The experiments that identified and delimited the P-domain (Gluschankof & Fuller, 1994) could not determine whether it forms part of the active site or has some other essential role in biosynthesis. The new crystal structures physically establish the P-domain as a true polypeptide domain (Holyoak *et al*., 2003; Henrich *et al*., 2003). As shown in Fig. 1, the P-domain is a closed 'thicket' of β-strands that contributes no amino acid to the active site or specificity pockets. Although the P-domain is located on the far side of the molecule relative to the amino terminus, the prodomain,

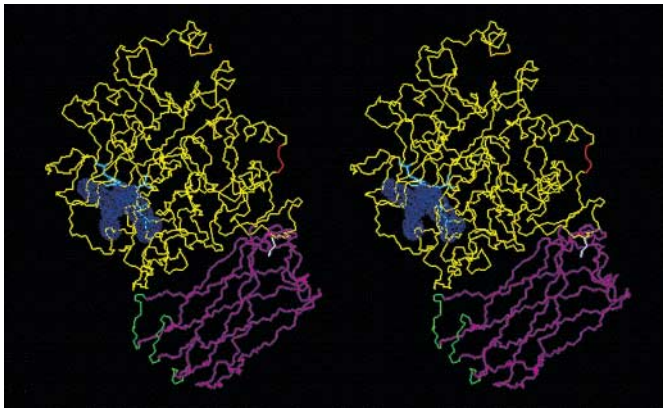

Fig. 1 | Crystal structure of Kex2 protease. Stereo image with the amino terminus in orange, subtilisin domain in yellow, active-site residues in cyan, inhibitory peptide in blue, P-domain in purple, and C terminus in white. The pro-domain (Tangrea *et al*., 2002) would have extended from the N terminus across the active site, potentially to interact with the green surface of the P-domain. A site that could be engineered to test the hypothesis that the P-domain is dispensible after pro-domain cleavage is shown in red. Source data: Holyoak *et al.* (2003).

before cleavage, must have extended the N terminus across the active site in the direction of the P-domain (Fig. 1). This suggests that the prodomain might interact with the P-domain during PC maturation. The most extreme maturation hypothesis—that the P-domain is dispensable after folding and prodomain cleavage—can now be tested by mutation of residues 459 and 460 to a moderately good cleavage site such as Thr-Arg (Bevan *et al*., 1997). Such an alteration would potentially direct intermolecular autolysis of the P-domain from the subtilisin domain. Too favourable a site could result in inappropriately ordered biosynthetic cleavages (Anderson *et al*., 2002).

Analysis of secreted, soluble forms of PCs with fluorigenic peptide substrates has shown that the principal specificity pockets of Kex2 recognize Arg at P1 and Lys at P2 (Brenner & Fuller, 1992; Rockwell *et al*., 1997), with some substrate recognition at P4 (Rockwell & Fuller, 1998). Furin, conversely, favours Arg at P1, P4 and P6 (Molloy *et al*., 1992; Bravo *et al*., 1994; Jean *et al*., 1995; Krysan *et al*., 1999). As expected for a protease that recognizes substrate polypeptides as β-strands, the P1 Lys and P2 Arg of the bound inhibitors in the crystal structures are splayed out in opposite directions. Furthermore, the P1 Arg of peptide inhibitors bound to PCs is recognized not simply as a basic amino acid but specifically as Arg (Holyoak *et al*., 2003; Henrich *et al*., 2003). Asp 277 (Kex2 numbering) shows bidentate recognition of the P1 Arg εN and one of the η nitrogens. The other η nitrogen is coordinated by the carbonyl oxygens of residues 311 and 312, whereas Asp 325 coordinates both η amino groups from below. To rigidify the position of Asp 277 in the P1 pocket, Asp 277, Asp 320 and Glu 350 coordinate a calcium ion that, in subtilases, is uniquely found in PCs.

Whereas PC specificity is due largely to K_m effects, it has long been appreciated that Kex2 (Brenner & Fuller, 1992) and furin (Bravo *et al*., 1994) have burst kinetics with favourable substrates and that, for Kex2, the >100-fold reduction in the rate of cleavage of a P1-Lys substrate versus the favoured P1 Arg is accompanied by a change from acylation (Arg) to hydrolytic deacylation (Lys) as the rate-determining step (Rockwell & Fuller, 2001a,b). As kinetic data indicate that the Arg-binding interaction negotiates the burst (Lys at P1 is not disfavoured in hydrolysis), future structures that incorporate a nonfavoured P1 Lys are expected to reveal the subtle differences in geometry that are less productive for acylation.

To summarize, the structures of Kex2 and furin have revealed two inventions grafted onto the subtilisin framework: a β-strand-rich P-domain, the location of which suggests that it might interact with the prodomain before pro-PC maturation; and a P1-Arg-specificity pocket, with calcium and a constellation of side-chain and main-chain oxygens that position specific substrates for acylation with burst kinetics. The unique features of PCs, including a metal ion in the P1 pocket, suggest novel strategies for inhibiting these medically important enzymes.

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